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Use of Tissue Culture Methods in Screening for Anthracnose (Colletotrichum Fragariae Brooks) Resistance in Strawberry.

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USE OF TISSUE CULTURE METHODS IN SCREENING FOR ANTHRACNOSE (*Colletotrichum fragariae* Brooks) RESISTANCE IN STRAWBERRY

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
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in

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by

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Abstract: Use of Tissue Culture Methods in Screening for Anthracnose (Colletotrichum fragariae Brooks) Resistance in Strawberry

Calli derived from 'Tangi' (susceptible) and MSUS 42 and LA 8318 (resistant) exhibited differential responses when inoculated with Colletotrichum fragariae, isolate CF1. The best differential in terms of growth and multiplication of the fungus was observed at 15°C and this differential decreased with increasing incubation temperature and time. Level of benzyladenine in the medium did not have an effect on disease expression.

Susceptible tissues exhibited extreme sensitivity, characterized by intense discoloration and cessation of growth when subcultured onto a medium containing the culture filtrate of C. fragariae while the resistant tissues were affected less. The efficacy of the culture filtrate was not affected by autoclaving. The culture filtrate also induced more electrolyte leakage from susceptible tissues than from resistant tissues. These observations suggest that C. fragariae produces a toxin-like substance in vitro.

In vitro disease ratings of eight cultivars/lines were made based on callus response to inoculation with a conidial suspension of the fungus and exposure to culture

filtrate of the pathogen. MSUS 42, LA 8318, LA 6261 and 'Apollo' were classified as resistant, 'Earlibelle' as intermediate, and 'Tangi', 'Sunrise', and LA 42311 as susceptible. In vitro screening results correlate well ($r = 0.812$) with whole plant screening responses.

INTRODUCTION

Anthracnose, caused by Colletotrichum fragariae Brooks, is a serious disease of strawberry (Fragaria x ananassa Duch.) in the warm, humid summer conditions in the southeastern United States [15]. The fungus attacks the stolons, petioles, leaves, fruits and crowns and can cause wilting and sudden death of the infected plants [14]. The crown rot phase is the most devastating stage of the disease. C. fragariae does not overwinter in the soil but it can survive in infected strawberry plants [14], in coffeeweed (Cassia obtusifolia L.), and probably other weeds [15] which can serve as the source of inoculum the following spring.

Histopathological work done by Milholland [20] showed that in the susceptible cultivar 'Surecrop', intercellular and extracellular growth of the fungus in the cortex and vascular tissues resulted to cellular collapse and necrosis. In resistant cultivars 'Apollo' and 'Sequoia', the fungus was confined to a few cells beneath the infection site. Cell wall thickening, deposition of pectic materials in the intercellular spaces of the cortex and tannin accumulation in the parenchyma cells were associated with the restriction of fungal growth in the resistant cultivars [20].

Breeding for anthracnose resistance is a major objective in the strawberry improvement program and so

techniques for the identification of sources of resistance genes will facilitate the development of resistant cultivars. At present, screening for anthracnose resistance is accomplished by inoculation of test plants with conidial suspension of the fungus either as a plant spray [3, 24, 25] or as crown drops [24]. Tissue culture as a research tool may offer a novel technique for in vitro screening as long as resistance can be expressed by tissues in culture. It can also contribute to the elucidation of the mechanism of resistance.

Manipulations with cultured plant materials now supplement research performed at the whole plant level. Some applications of tissue culture include: 1) rapid propagation of superior cultivars, 2) rapid production of homozygous lines, 3) possibility of wide crosses via protoplast fusion, 4) increased variability in the germplasm base due to somaclonal variation, 5) in vitro germplasm exchange, thus limiting the introduction of pathogens into the recipient country, 6) selection of stress tolerant cell lines and the study of physiological processes involved with stress, and 7) elucidation of host-parasite interaction.

In plant pathological research, tissue culture offers additional ways to study host-parasite interaction with the following advantages [21]: 1) precise control of

the environment, 2) exclusion of extraneous organisms, 3) inoculation without wounding, 4) control of inoculum level and number of host cells, 5) ability to alter the nature of host-parasite interaction by varying the components of the growth medium, 6) presence of only one or few host cell types, and 7) ease of application and removal of materials such as labelled precursors from cultured cells.

This technique, however, also has certain disadvantages [9]: 1) the role of defensive barriers such as hairs, cuticle, or preexisting inhibitors may not be present in tissue culture system, 2) defenses built as a result of intercellular communication may be impossible to examine, 3) the events that occur at the whole plant level may be simply not the same as those in tissue culture and vice-versa, and 4) some genetic changes can occur in culture and if these changes result in a new and abnormal gene expression, then the results may not reflect the actual disease expression.

REVIEW OF LITERATURE

As background information, host-parasite interaction in vitro, factors affecting disease expression in vitro, and applications of tissue culture in pathological and crop improvement programs will be reviewed.

Host-Parasite Interaction In Vitro

Cell culture techniques may be utilized in the study of host-parasite interaction. They provide simple models for host-parasite relationships [17]. Ingram and Robertson [18] showed that tissue cultures of potato variety 'Majestic' (susceptible) supported good growth of Phytophthora infestans race 4 whereas 'Orion' (resistant) did not. Observations made on intact plants corresponded with in vitro results. Behnke [1] regenerated potato plants from callus resistant to the culture filtrates of P. infestans and found that the resistance of potato leaflets to culture filtrate was correlated with lower growth but not with lower sporulation.

The applicability of tissue culture system in the study of host-parasite interaction is best demonstrated in tobacco. Resistance to race 0 of P. parasitica var. nicotianae (Ppn) was expressed in tissue culture [2, 11, 12, 19]. Calli derived from susceptible plants were susceptible while those from resistant plants were resistant, indicating that the monogenic factor

conferring resistance to intact plants was expressed in callus cultures. Electron microscopy revealed that 24 hours after inoculation, a hypersensitive reaction was evident in the incompatible combination where almost all of the cells collapsed in contrast to the compatible combination where the cells remained turgid after 48 hours [4]. There was a high correlation between disease expression at the tissue culture level and the whole plant [2]. Pseudomonas tabaci, a bacterial pathogen of tobacco, multiplied rapidly and colonized the tobacco callus within two days after inoculation while P. pisi and P. fluorescens (both nonpathogens) multiplied slowly and remained on the inoculation sites [16]. Rates of bacterial multiplication obtained in vitro were comparable to those on inoculated leaves.

In soybean x P. megasperma var. sojae (Pms) system, calli derived from 'Cutler 71' (resistant) were less colonized by the fungus than those derived from 'Cutler' (susceptible) when both calli were grown on a medium containing 6 or 10 mg/l 2,4-D and incubated at 16 or 20°C prior to and following inoculations with Pms zoospores [13]. Experiments with tomato tissue culture demonstrated that at least partial resistance to race O of P. infestans was maintained in undifferentiated tissues [27]. This particular study did not determine if resistance to the fungus was due to changes in phenolic

metabolism because of the differences in the fractionation and chromatography of phenolics from the leaves. The alfalfa callus system developed by Miller et al. [22] demonstrated the expression of resistance and susceptibility to P. megasperma f. sp. medicaginis (Pmm) in vitro.

Factors Affecting Disease Expression In Vitro

Factors that affect disease expression in vitro are inoculation procedure, environmental conditions and balance of growth regulators [10]. Quantification of the inoculum and inoculation method are of critical importance. Tissues must be inoculated in such a way that there is no contact between the pathogen and the culture medium. This can be done by confining the inoculum in a piece of tygon tubing [10], using mycelial blocks [22], or transferring and inoculating the calli in sterile petri plates with several drops of water added to maintain a high relative humidity [2]. Precise quantification of the inoculum density cannot be done if mycelial blocks are used.

Environmental conditions may also be an important consideration. Temperature appears to be a critical factor. In tobacco x Ppn system, 20^{o}C gave the best differential between the susceptible and resistant genotypes [11]. At 28^{o}C, the differential broke down.

Light was not important here. In alfalfa x Pmm system, 21^{o}C was best [22] while in soybean x Pms system, it was 16^{o}C [13].

Growth regulators are essential in the growth of the tissues in culture and tissue morphology may influence the disease expression. Tobacco tissues exhibited genotype-specific resistance to Ppn when supplied with 11.5 uM indoleacetic acid and either 1 uM kinetin or benzyladenine but resistance broke down when the level of cytokinin was raised to 10 uM. Tight callus supported less growth of the fungus while loose, friable cells were susceptible regardless of their genotypes [11]. In soybean callus, maximum differential in the colonization by Pms occurred when the level of 2,4-D was 6 mg/l. Calli derived from the resistant variety were tighter and less friable [13]. Alfalfa calli grown on a medium with a high concentration of kinetin (4 mg/l) were friable and dry in appearance and as the concentration decreased, they appeared more fluid, favoring the growth of Pmm [22]. A kinetin concentration of 2 mg/l was necessary for the expression of resistance or susceptibility.

Applications of Tissue Culture in Pathological And Crop Improvement Programs

Tissue culture is now being utilized to supplement the present effort in crop improvement programs. In

vitro selection for resistance to various plant pathogens and subsequent regeneration have been employed in several economically important crops. The reaction of plant tissues in culture to the toxin produced by the pathogen and its correlation to the whole plant level response are expanding areas of research because of the convenience offered by this technique.

Cell lines of alfalfa (Medicago sativa) have been selected for resistance to the toxin(s) produced by Fusarium oxysporum f. sp. medicaginis by incorporating the culture filtrate of the pathogen into the callus medium [8]. Plants regenerated from the resistant cell cultures showed resistance to the pathogen.

In corn (Zea mays), selection of resistant materials from the susceptible T-cytoplasm callus cultures growing on a medium containing the HmT toxin followed by regeneration yielded resistant plants that were also resistant to Helminthosporium maydis race T at the field level [5, 7]. The regenerated plants from the toxin resistant callus did not show the T-male sterility trait so they were of no agricultural value. However, the mitochondrial DNA of the regenerated plants was not similar to the N-type but was a variant of the T-type mitochondrial DNA, indicating that a new type of resistant material was recovered [7].

Resistance of oat (Avena sativa) to victorin, the toxin produced by H. victoriae is a recessive trait. The approach of Gengenbach and Rines [6] made use of selections in cultures that had been initiated from heterozygous Vbvb toxin susceptible oat lines. Plants regenerated from toxin selection medium were insensitive to victorin as shown by the leaf bioassay. Inheritance studies proved that the regenerated toxin-insensitive plants were genetically stable [6].

Selection for toxin resistance in tissue cultures has resulted in improved disease resistance in regenerated plants. Potato plants regenerated from callus resistant to the culture filtrate of P. infestans exhibited 25% reduction in lesion size when inoculated with the fungus [1]. Reduced disease symptoms, characterized by smaller necrotic spots, were observed in inoculated rape plants (Brassica napus) regenerated from tissue cultures selected for Phoma lingam toxin resistance [23]. Regenerated tobacco plants from cultures resistant to AA-toxin likewise produced reduced disease symptoms on the leaves when inoculated with Alternaria alternata [26].

The application of tissue culture techniques in various aspects of plant research is a very promising area. In vitro screening of germplasm is not a distant possibility considering that the present trend of

international germplasm exchange is through cell culture. If procedures for specific crops can be established, this can be an efficient and convenient screening method.

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EXPRESSION OF RESISTANCE IN STRAWBERRY CALLUS INOCULATED WITH *Colletotrichum fragariae* Brooks

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Abstract:

Calli derived from susceptible ('Tangi') and resistant (MSUS 42 and LA 8318) strawberry plants exhibited differential response, when inoculated with *Colletotrichum fragariae* Brooks. The best differential in terms of growth and multiplication of the fungus on susceptible and resistant calli was expressed at 15°C. This differential decreased with increasing incubation time and temperature. The level of benzyladenine in the medium did not have an effect on disease expression.

Abbreviations: BA: N-(phenyl-methyl)-1-H-purine-6-amine, IBA: 1H-indole-3-butanoic acid, GA_3: gibberellic acid, NAA: 1-naphthaleneacetic acid, MS: Murashige and Skoog [9].

INTRODUCTION

Anthracnose, caused by Colletotrichum fragariae Brooks, is a serious disease of strawberry in the southeastern United States. The crown rot phase is the most devastating stage of the disease. Breeding for crown rot resistance is a major objective in the strawberry improvement program and techniques for the identification of sources of resistance genes will facilitate the development of new cultivars. Tissue culture as a research tool may augment the present effort of screening for crown rot resistance if it can be shown that disease expression can be manifested in vitro.

In vitro expression of resistance or susceptibility to pathogens has been reported in tobacco [2, 4, 5], potato [1, 7], alfalfa [8], soybean [6], and tomato [11]. In most cases disease expression was manifested by the degree of colonization of the callus by the pathogen. Calli derived from resistant plants were able to check the growth and multiplication of the pathogen, thus indicating resistance. Factors affecting disease expression in vitro include temperature and growth regulators [4, 5, 6, 8]. Each host-pathogen system requires a critical temperature, beyond which the differential may breakdown [4, 6]. Growth regulators that are essential in the growth of the tissues in culture may influence tissue morphology that may, in

turn, affect the colonization of the callus by the pathogen [5, 8]. The objective of this study was to determine the conditions conducive to proper disease expression of strawberry callus against C. fragariae.

MATERIALS AND METHODS

Establishment of Strawberry Tissue Culture

Runners of the susceptible cultivar 'Tangi' and resistant lines MSUS 42 and LA 8318 were obtained from Louisiana State University Agricultural Experiment Station in Baton Rouge, Louisiana. Sterilization was accomplished by immersing the shoots for 10 minutes in a 1.05% sodium hypochlorite solution (20% commercial bleach solution) containing a drop of Tween 20. The shoots were rinsed twice with sterile distilled water, the outer leaves were removed, then the shoots were reimmersed in 0.52% sodium hypochlorite solution for 5 minutes and finally rinsed three times with sterile distilled water. The shoot tips were established in 25 x 150 mm culture tubes containing modified MS medium supplemented with 1 mg/l BA, 1 mg/l IBA, 0.01 mg/l GA_3, 30 g/l sucrose, and 8 g/l agar. The pH of the medium was adjusted to 5.7 prior to sterilization (1 kg/cm^2, 121°C for 15 minutes). Calli formed on the shoots were transferred into another medium containing the MS basal salts supplemented with

1 mg/l NAA, 1 mg/l BA, 100 mg/l myo-inositol, 10 mg/l thiamine, 30 g/l sucrose, and 8 g/l agar.

Culture of the Pathogen and Preparation of Inoculum

Culture of C. fragariae, isolate CF1, was obtained from Dr. Barbara Smith, USDA, Poplarville, Mississippi and was preserved in silica gel [10]. Fungal cultures were initiated by placing several silica crystals on petri plates containing a mixture of Difco potato dextrose agar (19.5 g/l) and Difco oatmeal agar (30 g/l). Fungal plates were incubated at 25°C under continuous cool white fluorescent light (100 uE m^{-2} sec^{-1}). Inoculum was prepared by flooding 10-14 day old plates with sterile distilled water, filtering through cheesecloth and diluting with water until the desired conidial concentration was reached. Normally, 20 ul of the prepared suspension delivered 20-25 conidia.

Inoculation and Incubation

Three to four week-old calli, subcultured in scintillation vials containing 10 ml callus medium, were preincubated at 15, 20, and 25°C for at least 24 hours prior to inoculation. A piece of tygon tubing (3/16" diameter) was placed on top of each callus to contain the inoculum. Inoculated calli were incubated at their respective temperatures and rated daily using the

following rating system [8]: 0 = no aerial hyphae visible, 1 = aerial hyphae on upper 25% of callus, 2 = aerial hyphae on upper 50% of callus, 3 = aerial hyphae on upper 75% of callus, and 4 = aerial hyphae on entire callus. Sporulation was estimated by taking a portion of the mycelia (25 mm^2) from 3 random samples from each treatment at the end of the experiment, triturating it in 0.5 ml distilled water and taking a spore count using a hemacytometer. Data from the temperature study were expressed as percentage of 'Tangi'.

To determine the effect of BA on disease expression, 'Tangi' and MSUS 42 calli were subcultured in the callus medium containing various levels of BA (0.1, 0.5, 1.0, 2.0, 3.0, and 4.0 mg/l). The samples were preincubated at $15^{o}C$ prior to inoculation with the conidial suspension, returned to the same incubation temperature after inoculation, and the fungal growth was rated daily.

Ten tubes per treatment combination were used. All experiments were repeated 3-4 times, each repetition serving as a replicate. Data that were expressed in percentage were transformed using arcsine transformation and analyzed using the transformed values.

RESULTS

Effect of Temperature

Inoculated calli of 'Tangi', MSUS 42, and LA 8318, incubated at 15, 20, and 25°C showed that the best differential based on fungal growth rating was observed at the 2 lower temperatures. At 25°C, the fungus colonized the MSUS 42 calli as rapidly as the 'Tangi' calli and both were almost completely colonized on the fifth day after inoculation (Fig. 1, Table 1). On the seventh day at 15°C, the fungus grew 30% less on MSUS 42 calli as compared to the susceptible check 'Tangi'. LA 8318 calli were colonized less by the fungus at all 3 temperatures. In all cases, the observed differential narrowed down as the incubation temperature and time increased.

Fungal sporulation increased as the temperature increased in all 3 cultivar/lines (Fig. 2, Table 2). The best differential in terms of sporulation was observed at 15°C. The fungus sporulated comparatively less on LA 8318 and MSUS 42 than on 'Tangi'. Sporulation rate on the resistant and susceptible calli at 20 and 25°C can not be clearly differentiated.

Effect of Benzyladenine

Fungal growth on 'Tangi' and MSUS 42 at 15°C was not affected by the level of BA in the medium (Fig. 3).

There were no significant differences among the various BA levels within the cultivar/line but fungal growth rating between 'Tangi' and MSUS 42 was significant regardless of the BA level used. No appreciable change in tissue morphology was noted among the treatments.

DISCUSSION

Disease expression in vitro can be manifested if proper conditions are provided to tissues in culture. The best incubation temperature for the strawberry x *C. fragariae* system is 15°C while BA concentration is not critical for disease expression. As in tobacco x *Phytophthora parasitica* var. *nicotianae* [4], soybean x *P. megasperma* var. *sojae* [6] systems and possibly other host-pathogen in vitro interactions, increasing the incubation temperature causes the breakdown of any observed differential between the tissues derived from resistant and susceptible plants. Although the cytokinin concentration has been reported to influence in vitro disease expression [3, 8], such an effect was not demonstrated in this study.

Resistance of a plant to a pathogen is indicated by its ability to check the growth and multiplication of the pathogen. Results obtained from this in vitro study, namely, lesser growth and sporulation of the fungus on LA 8318 and MSUS 42 suggest that calli derived from

resistant strawberry lines were able to manifest this criterion. Reduced colonization of the resistant tissues by the pathogen was also reported in alfalfa [8], tobacco [4, 5], tomato [11], potato [7], and soybean [6]. The mechanism of resistance of certain strawberry cultivars and lines to C. fragariae is not fully understood but the data obtained from this study may perhaps serve as a model to partially explain the basis of resistance at the whole plant level. Further studies leading to the elucidation of the molecular basis of such resistance warrant serious investigation.

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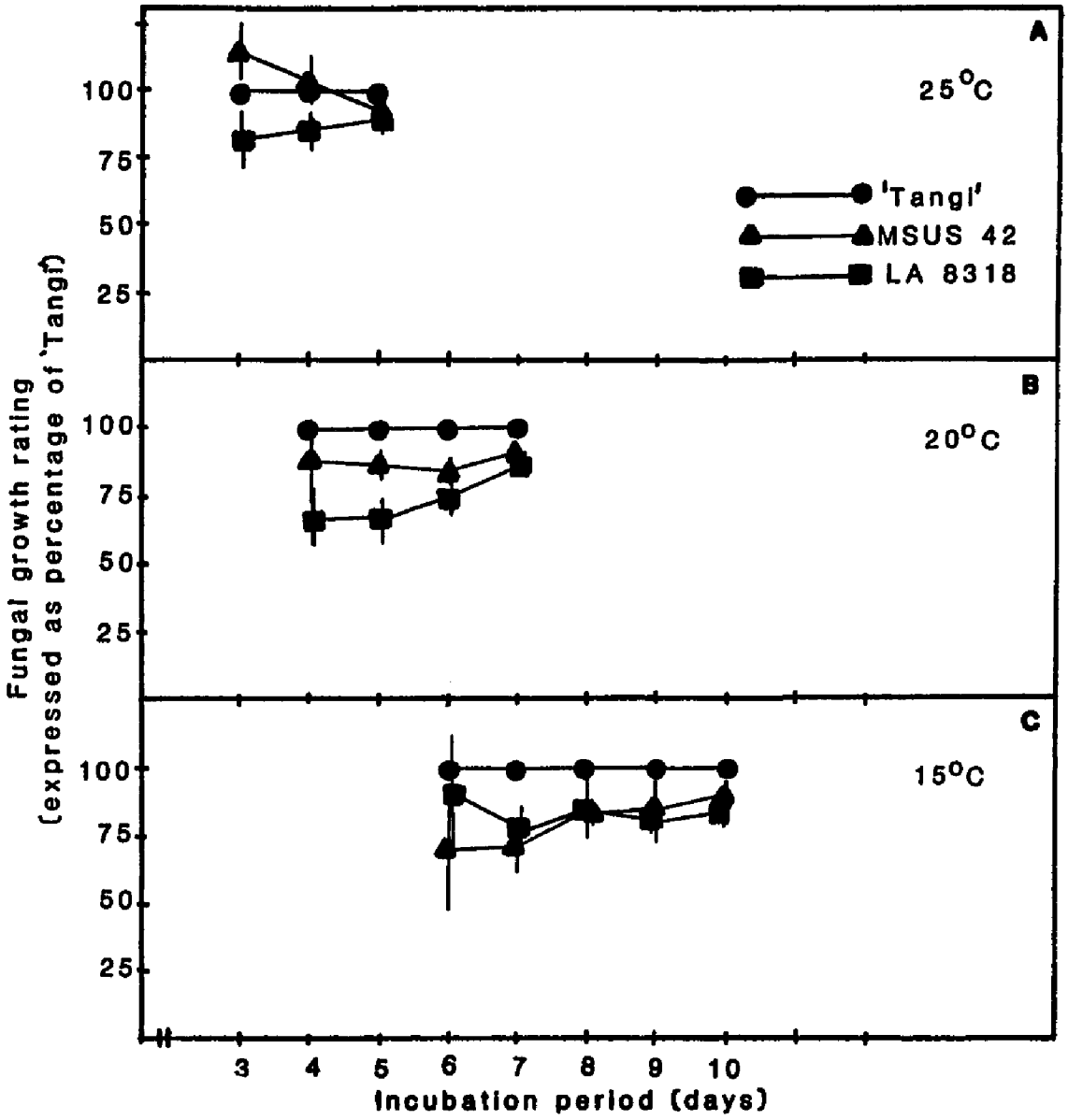

Fig. 1. Growth of C. fragariae, isolate CF1, on susceptible ('Tangi') and resistant (MSUS 42 and LA 8318) strawberry calli incubated at A = 25°C, B = 20°C, and C = 15°C. Rating system: 0-4; 0 = no aerial hyphae visible, 4 = aerial hyphae on entire callus. Data are expressed as percentage of 'Tangi'. Bars represent standard deviations.

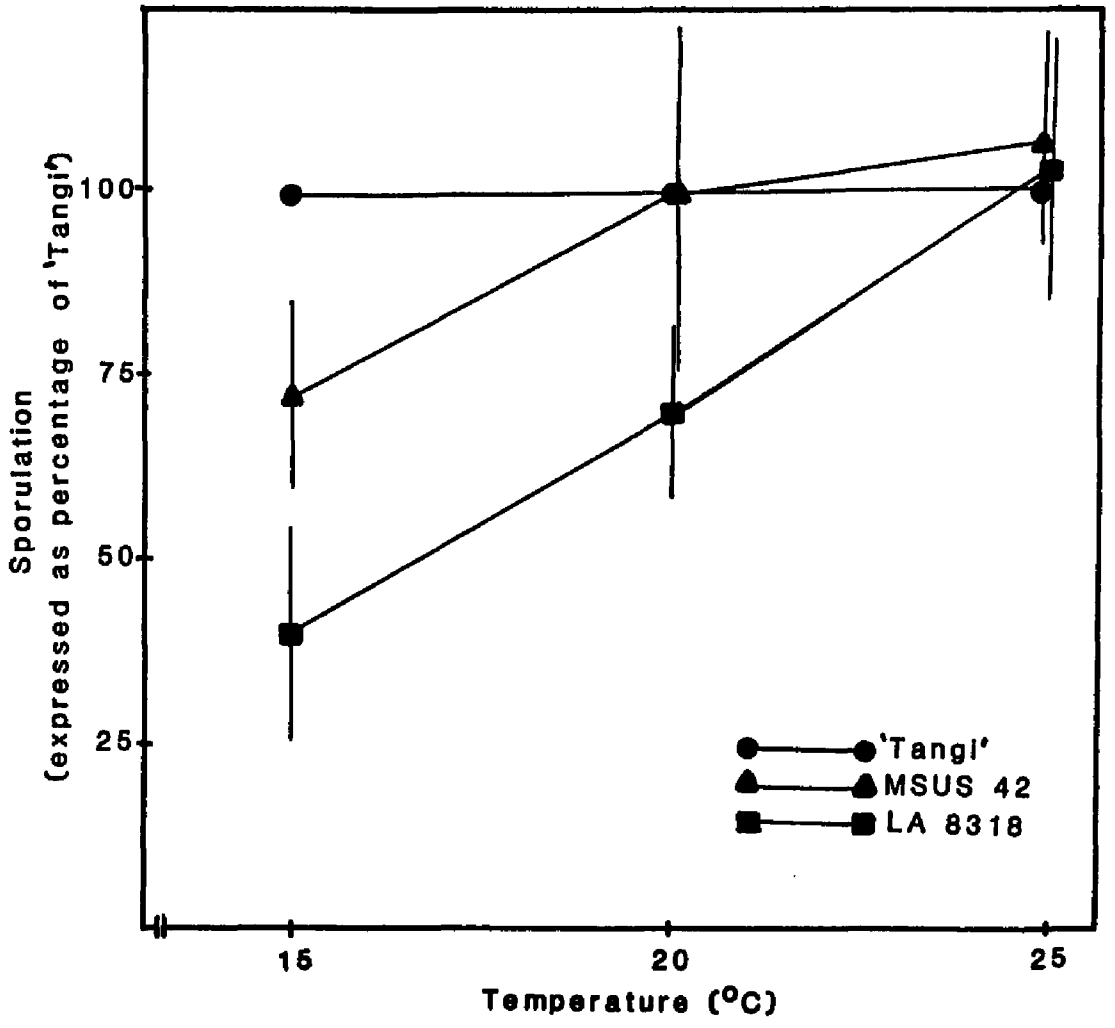

Fig. 2. Sporulation of C. fragariae, isolate CF1, on susceptible ('Tangi') and resistant (MSUS 42 and LA 8318) strawberry calli incubated at 25, 20, and 15°C. Data are expressed as percentage of 'Tangi'. Bars represent standard deviations.

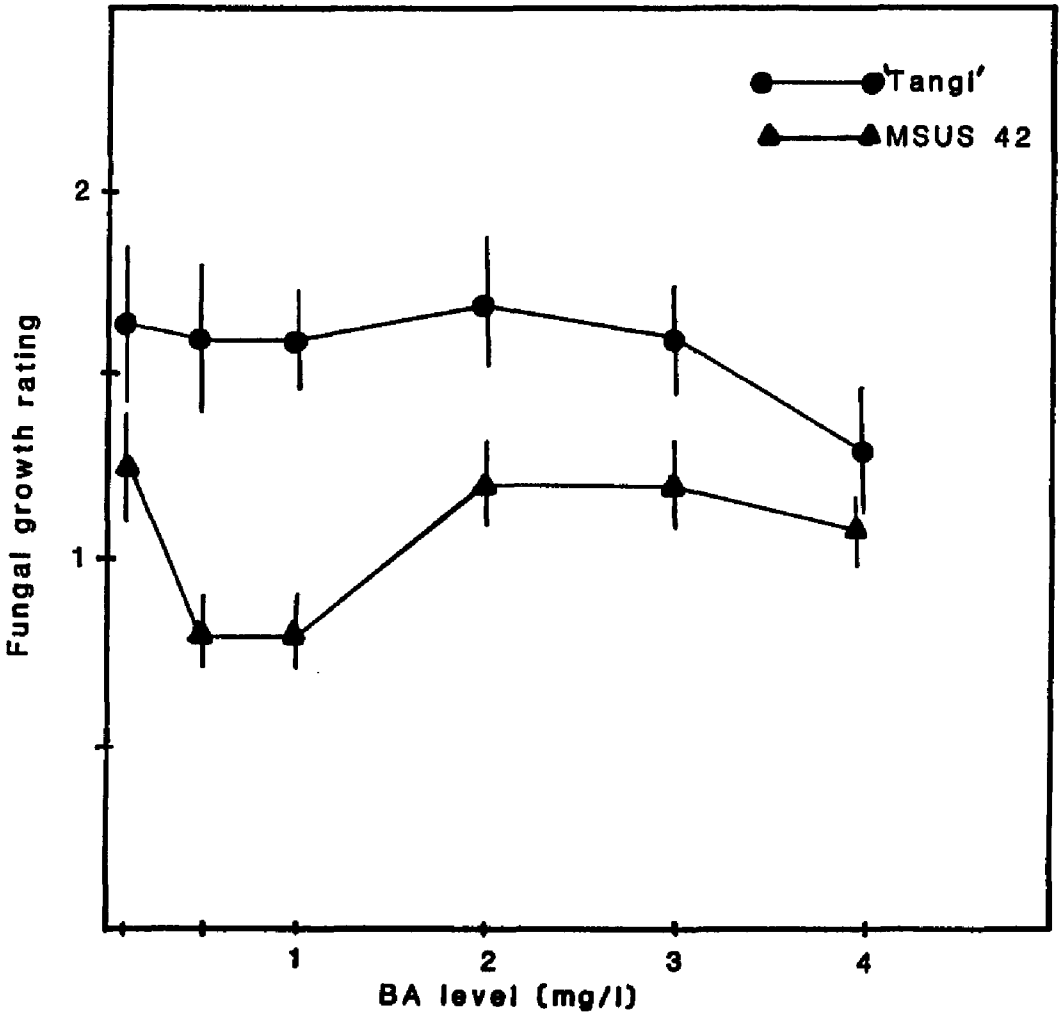

Fig. 3. Growth of C. fragariae, isolate CF1, on susceptible ('Tangi') and resistant (MSUS 42) strawberry calli subcultured on modified MS medium with various BA concentrations, incubated at 15°C, 7 days after inoculation. Rating system: 0-4, 0 = no visible aerial hyphae, 4 = aerial hyphae on entire callus. Bars represent standard deviations.

Table 1. Growth of C. fragariae, isolate CF1, on susceptible ('Tangi') and resistant (MSUS 42 and LA 8318) strawberry calli[z].

Temp. (°C)	Cultivar/ Line	Incubation Period (days)							
		3	4	5	6	7	8	9	10
25									
	'Tangi'	1.2(0.1)	2.6(0.1)	3.7(0.1)					
	MSUS 42	1.4(0.1)	2.7(0.1)	3.4(0.1)					
	LA 8318	1.0(0.1)	2.2(0.1)	3.3(0.2)					
20									
	'Tangi'		1.3(0.1)	2.4(0.1)	3.4(0.1)	3.9(0.1)			
	MSUS 42		1.1(0.2)	2.1(0.1)	2.9(0.1)	3.5(0.1)			
	LA 8318		0.9(0.2)	1.6(0.1)	2.5(0.2)	3.4(0.2)			
15									
	'Tangi'				0.6(0.1)	0.9(0.1)	1.5(0.1)	2.3(0.1)	2.8(0.1)
	MSUS 42				0.4(0.1)	0.6(0.1)	1.3(0.1)	1.9(0.2)	2.5(0.1)
	LA 8318				0.5(0.1)	0.7(0.1)	1.3(0.1)	1.7(0.1)	2.2(0.1)

[z]Average of 5 replications with 10 calli per replication; values in parentheses represent standard deviations.

Table 2. Sporulation of C. fragariae, isolate CF1, on susceptible ('Tangi') and resistant (MSUS 42 and LA 8318) strawberry calli.

Incubation Temperature (°C)	Cultivar/ Line	Spore count (10^3)
25	'Tangi'	43.02 (9.16)[z]
	MSUS 42	42.60 (4.83)
	LA 8318	36.82 (8.23)
20	'Tangi'	25.62 (4.13)
	MSUS 42	24.12 (4.65)
	LA 8318	18.85 (5.33)
15	'Tangi'	13.12 (3.18)
	MSUS 42	8.85 (1.83)
	LA 8318	6.62 (4.06)

[z]Average of 4 replications with 3 random samples per treatment combination; estimated by triturating 25 mm^2 mycelia in 0.5 ml water and making a spore count using a hemacytometer; values in parentheses represent standard deviations.

RESPONSE OF STRAWBERRY CALLUS TO CULTURE FILTRATE OF *Colletotrichum fragariae* Brooks

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Abstract:

Calli derived from susceptible cultivar 'Tangi' exhibited extreme sensitivity, characterized by intense discoloration and cessation of growth when subcultured in a medium containing the culture filtrate of *Colletotrichum fragariae*, isolate CF1. Those derived from resistant lines MSUS 42 and LA 8318 were affected less by the applied treatments. The efficacy of the culture filtrate was not affected by autoclaving. The culture filtrate also induced more electrolyte leakage in the susceptible tissues than in the resistant tissues. The possibility that the fungus produces a toxin-like substance was discussed.

Abbreviations: BA: N-(phenyl-methyl)-1-H-purine-6-amine, NAA: 1-naphthaleneacetic acid, MS: Murashige and Skoog [15].

INTRODUCTION

The role of pathogen-produced toxins in disease development is well documented [20, 26, 27]. Toxins are active at very low concentrations [6]. Extraction from diseased plants can cause a problem since these substances may exist in very minute amounts, they may be very unstable, or they may be bound or inactivated in plant tissues [21], making isolation and characterization difficult.

Many pathogens growing in vitro secrete substances which when introduced into the host plant reproduce some or all of the symptoms associated with infection by that pathogen [5]. These substances may cause the disruption of metabolic processes in the host and are therefore suspected to be involved in pathogenesis. A number of toxins have been isolated from culture filtrates of the pathogens. Colletotin, a toxin produced by Colletotrichum fuscum, induced the wilting symptom in Digitalis lanata and D. purpurea [11]. The sensitivity to two toxic substances (HC toxin and carbtoxinine) isolated from the culture filtrates of Helminthosporium carbonum was correlated with susceptibility of corn to that pathogen [18]. The susceptibility of sugarcane clones to H. sacchari was also correlated with their sensitivity to HS toxin that was isolated from the culture filtrate of the fungus [23]. Other toxins

isolated from the culture filtrates of the pathogens that are involved in disease development include T-toxin from H. maydis [9], victorin from H. victoriae [19], AK toxin from Alternaria kikuchiana [16], and AA toxin from A. alternata f. sp. lycopersici [10].

In vitro selection for resistant cell lines using toxins and subsequent regeneration of the resistant cell lines are now being used to augment conventional breeding methods. Selection of T-toxin resistance in tissue cultures of corn resulted in the isolation of many resistant cell lines and many regenerated plants were resistant [7, 8, 9]. In certain cases, this is an alternative approach especially if resistance is a recessive trait as in the case of oat x H. victoriae [8]. Promising results have been reported in tobacco x Pseudomonas syringae pv. tabaci [4], potato x Phytophthora infestans [3], alfalfa x Fusarium oxysporum [12], and sugarcane x H. sacchari [13].

In view of these present trends in the application of tissue culture techniques in crop improvement, this study was initiated to meet the following objectives: 1) to demonstrate the possibility that C. fragariae produces a toxin-like substance in the culture filtrate; 2) to test the reaction of strawberry callus to the culture filtrate; and 3) to utilize the "toxin principle" as a

potential selection agent in selecting for resistance in vitro.

MATERIALS AND METHODS

Production of Culture Filtrate

Isolate CF1 of Colletotrichum fragariae was obtained from Dr. Barbara Smith, USDA, Poplarville, Mississippi, and was preserved in silica gel [17]. Fungal cultures were initiated by placing several silica crystals onto petri plates containing a mixture of Difco potato dextrose agar (19.5 g/l) and Difco oatmeal agar (30 g/l). The plates were incubated at 25°C under continuous cool white fluorescent light (100 uE m^{-2} sec^{-1}). One liter flasks containing 200 ml of sterilized liquid Czapek-Dox medium [24] were inoculated with 20 mycelial plugs (15 cm^2) taken from 7-10 day old fungal cultures. Inoculated flasks were incubated in the dark at 25°C. Culture filtrate was harvested at 10-day intervals by sequentially filtering through several layers of cheesecloth, Whatman #42 filter paper, Seitz filter (0.45 u), and finally filter sterilized using the Nalgene filter unit (0.22 u). The culture filtrate was stored in the freezer (-4°C) until required.

The use of conidial suspension as inoculum in the production of culture filtrate was also evaluated. Fifty ml of sterilized liquid Czapek-Dox medium contained in

250 ml flasks were inoculated with a conidial suspension to give a final concentration of approximately 5 x 10^4 conidia/ml. Inoculated flasks were incubated on a shaker (100 rpm) at room temperature. Culture filtrate was harvested at 5-day intervals filtered and stored as above.

Preparation of Culture Filtrate-Containing Medium

Callus medium (MS basal salts supplemented with 1 mg/l BA, 1 mg/l NAA, 100 mg/l myo-inositol 10 g/l thiamine, 30 g/l sucrose and 8 g/l agar) containing the desired amounts of culture filtrate (0, 6.25, 12.5, 25, and 50%, by volume) was prepared by replacing water with an equivalent amount of filter sterilized culture filtrate that was added to the autoclaved medium when it had cooled down to 35^{o}C. Similarly stored uninoculated Czapek-Dox was used as control. In a second experiment, the culture filtrate (50% level) was added to the medium prior to autoclaving (1 kg/cm^2, 121^{o}C for 15 minutes). Fifteen ml of the prepared medium were dispensed into petri plates onto which callus pieces of MSUS 42 and LA 8318 (both resistant) and 'Tangi' (susceptible) were transferred aseptically. Four plates with four calli per plate were used per treatment combination. Calli were rated after 30 days using the following rating system: 0 = profuse callus growth, no discoloration; 1 = moderate

callus growth, no discoloration; 2 = slight callus growth, no discoloration; 3 = very slight callus growth, 50% discoloration; and 4 = no growth, 100% discoloration.

Electrolyte Leakage

One gram calli of LA 8318 (resistant) and 'Tangi' (susceptible) were treated with 10 ml culture filtrate contained in 50 ml Erlenmeyer flasks with flasks containing Czapek-Dox medium as controls. The flasks were incubated on a shaker (100 rpm) at room temperature for 12 hours. The cells were washed and resuspended in deionized water following a procedure modified from Scheffer's [22]. Hourly electrolyte leakage was measured as specific conductance (umhos/cm^3) using a conductivity bridge (Amber Science Model 1051). The data were expressed as specific conductance of treated minus specific conductance of control, expressed as percentage of initial readings at time 0. Three replicates per treatment combination were used and the experiment was repeated twice. The same trend was observed in each experiment so the data from both experiments were combined.

RESULTS

Effect of Incubation Period On Culture Filtrate Production

Both susceptible and resistant calli exhibited slight growth with little discoloration in the medium containing 50% culture filtrate initiated from mycelial plugs harvested 10 days after inoculation (Table 1). Incubation periods of 20 or more days, however, produced culture filtrates that caused intense discoloration in 'Tangi' calli but not in the LA 8318 and MSUS 42 calli. Succeeding experiments made use of culture filtrate produced in this manner and harvested between 3 and 4 weeks.

The use of conidial suspensions as inoculum and incubation of the fungal cultures on a shaker hastened the production of substances with toxin-like activity in the culture filtrates capable of causing discoloration of the susceptible tissues. Culture filtrates harvested 10 and 15 days after inoculation resulted in substantial discoloration of 'Tangi' calli, however, an intense discoloration occurred when 20-day old culture filtrates were utilized (Table 2).

Effect of Autoclaving on the Efficacy of Culture Filtrate

Autoclaving the culture filtrate apparently did not affect its efficacy. The susceptible tissues showed

intense discoloration and cessation of growth in the culture medium containing either autoclaved or filter sterilized culture filtrate. LA 8318 and MSUS 42 calli showed sign of growth without discoloration in both treatments. These results indicate that the "toxic components" of the culture filtrate are not heat labile.

Effect of Culture Filtrate on Callus Growth

Filter sterilized culture filtrate added to the medium at various levels affected the growth of the strawberry callus. The highest concentration of culture filtrate used (50%) caused intense discoloration and death of the 'Tangi' calli and this effect diminished with decreasing culture filtrate concentrations in the medium (Fig. 1 and 2). Culture filtrate levels lower than 12.5% did not produce any significant reduction in growth of 'Tangi' calli. MSUS 42 calli were affected less by the culture filtrate treatment. Although the resistant calli grew slowly at the two higher concentrations, they did not show any discoloration, characteristic of the reaction of susceptible tissues to the culture filtrate. Both MSUS 42 and 'Tangi' calli responded similarly to the control treatments that made use of corresponding amounts of Czapek-Dox medium instead of the culture filtrate. A fifty percent level of the culture filtrate was used in the subsequent experiments.

Electrolyte Leakage

Electrolyte leakage was greater in the susceptible as compared to the resistant tissues (Fig.3). Electrolyte leakage was evident within the first two hours after treatment. Treated 'Tangi' tissues lost 44% more electrolytes than the control during the first hour and this loss increased to 63% during the next hour. Discoloration in the treated susceptible tissues was also observed. LA 8318 tissues on the other hand, lost comparatively less electrolytes during the first three hours (20-21% more than the control) and this loss decreased eith time. It was evident that the culture filtrate induced more electrolyte leakage in the susceptible tissues.

DISCUSSION

The results indicate that C. fragariae produces a toxin-like substance in vitro to which the tissues derived from susceptible plants are sensitive to. This was evidenced by the ability of the culture filtrate to cause the discoloration and death of 'Tangi' calli but not of MSUS 42 and LA 8318 calli. Rapid electrolyte loss induced by the culture filtrate in susceptible tissues is also an added proof to this hypothesis. One of the first symptoms of pathogen-induced damage to plant cells is alteration in membrane permeability and consequently

electrolyte leakage. Such changes in membrane permeability can be caused by the pathogen-produced toxin that may affect the membrane-bound enzymes [14], the components of the transport system [1, 2], or it may disrupt the energy supply for the maintenance and repair of the membrane [25]. Maintenance of ionic balance within the cell is very important for normal cellular processes and any disruption in this balance can be detrimental.

Since it was shown that the culture filtrate does more damage to the susceptible tissues than the resistant tissues, its potential use as a selection agent to distinguish resistant cell lines in vitro becomes possible. This technique has been used in corn, alfalfa, tobacco, oat and other economically important crops [3, 7, 8, 12]. The incorporation of partially purified HmT toxin in the T-cytoplasm corn callus culture medium and selection in subsequent subcultures produced regenerated plants that were toxin resistant and had increased pathogen resistance at the field level [7, 8]. They were of no agricultural value because the Texas-type male sterility was no longer shown but it demonstrated that such technique was possible. The reaction of 182 sugarcane clones to partially purified HS toxin obtained from the culture filtrate of the pathogen significantly

correlated to their reaction to H. saccharí [22], thus demonstrating the value of toxins in screening work.

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Table 1. Effect of incubation time of culture filtrate (CF) derived from mycelial plugs (15 cm^2) on callus discoloration of three strawberry cultivar/lines.

Cultivar/Line[z]	Incubation time (days)[z] 10	20	30
'Tangi'			
CF	2.4 (0.2)[y]	4.0 (0)	4.0 (0)
Control	1.0 (0)	0.4 (0.3)	1.0 (0)
MSUS 42			
CF	2.0 (0)	2.0 (0)	2.0 (0)
Control	1.0 (0)	0.9 (0)	1.0 (0)
LA 8318			
CF	2.0 (0)	2.0 (0)	2.0 (0)
Control	0.8 (0.2)	0.8 (0.2)	1.0 (0)

[z]Cultivar/line and incubation time highly significant ($P<0.01$); control not included in the analysis; data taken 30 days after subculture in a medium containing 50% culture filtrate.

[y]Rating system: 0 = profuse callus growth, no discoloration; 1 = moderate callus growth , no discoloration; 2 = slight callus growth, no discoloration; 3 = very slight callus growth, 50% discoloration; 4 = no growth, 100% discoloration; values in parentheses are standard deviations.

Table 2. Effect of incubation time of culture filtrate (CF) derived from conidial suspension as inoculum (5×10^4 conidia/ml) on callus discoloration of three strawberry cultivars/lines.

Cultivar/line[z]	Incubation time (days)[z]			
	5	10	15	20
'Tangi'				
CF	2.7 (0.2)[y]	3.4 (0.2)	3.4 (0.1)	4.0 (0)
Control	0.9 (0.2)	1.2 (0.2)	0.4 (0.3)	1.0 (0)
MSUS 42				
CF	2.1 (0.1)	2.0 (0)	2.0 (0)	2.0 (0)
Control	1.0 (0)	1.0 (0)	1.0 (0)	1.0 (0)
LA 8318				
CF	2.0 (0)	2.0 (0)	2.0 (0)	2.0 (0)
Control	1.0 (0)	1.0 (0)	1.0 (0)	1.0 (0)

[z]Cultivar/line and incubation time highly significant ($P<0.01$); control not included in the analysis; data taken 30 days after subculture in a medium containing 50% culture filtrate.

[y]Rating system: 0 = profuse callus growth, no discoloration; 1 = moderate callus growth, no discoloration; 2 = slight callus growth, no discoloration; 3 = very slight callus growth, 50% discoloration; 4 = no growth, 100% discoloration; values in parentheses represent standard deviations.

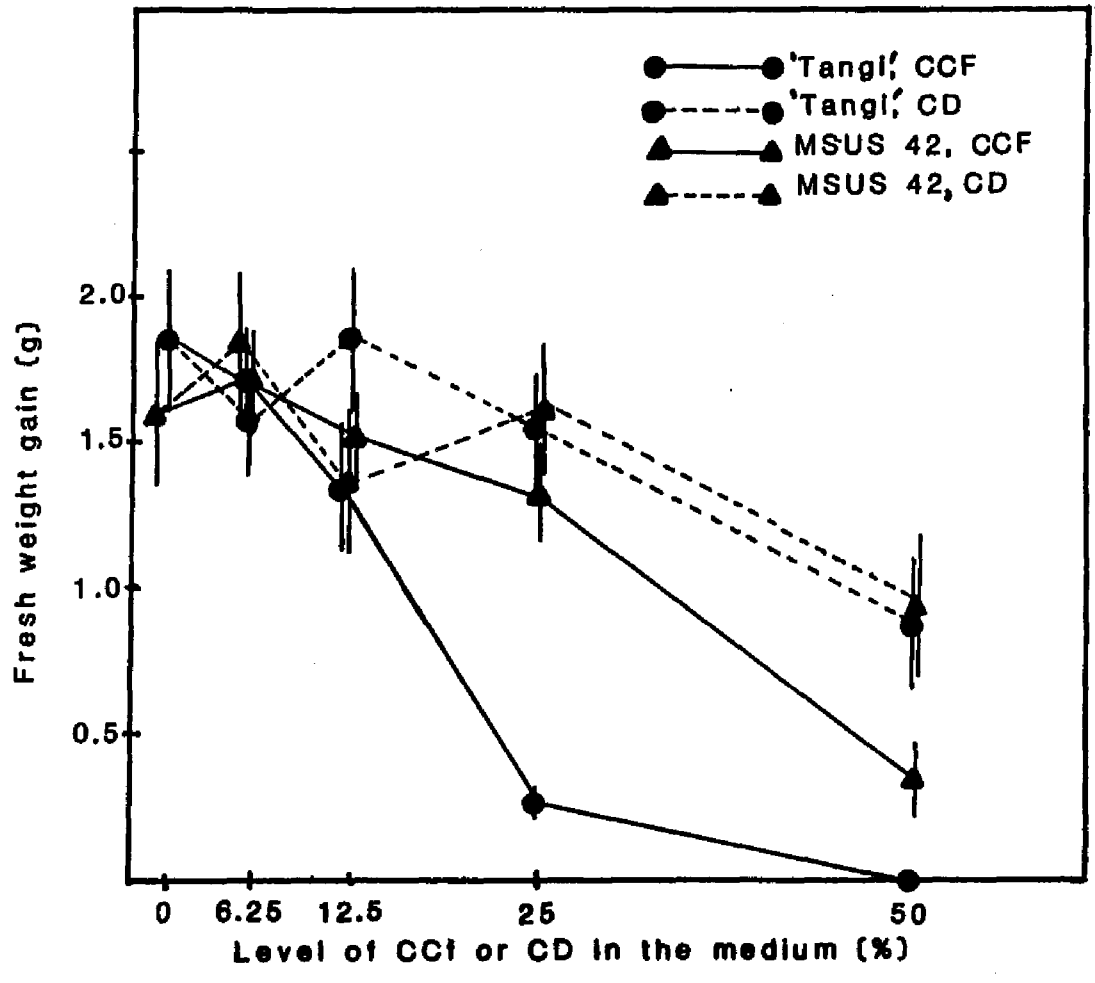
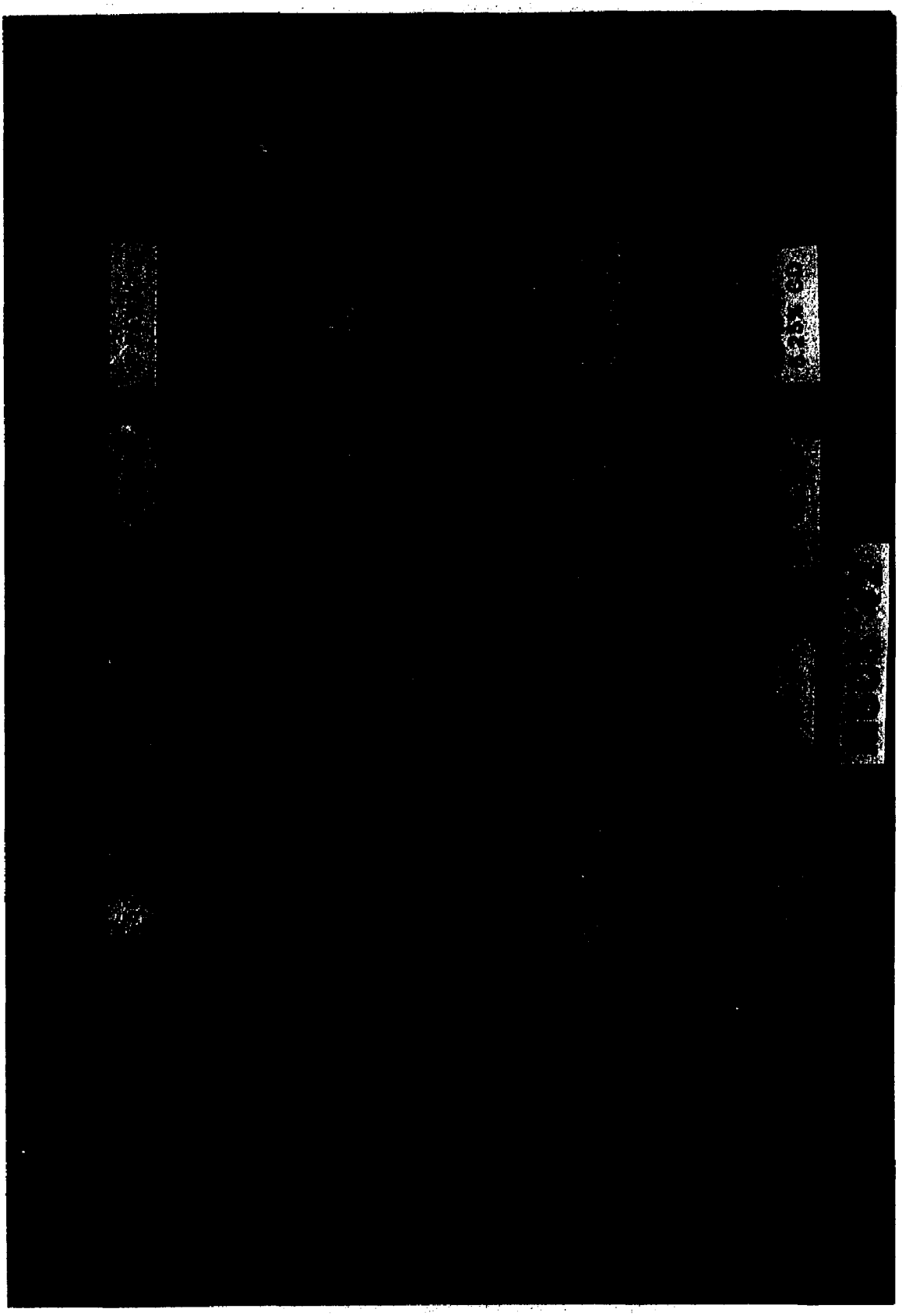


Fig. 1. Effect of culture filtrate of C. fragariae, isolate CF1, on the growth of susceptible ('Tangi') and resistant (MSUS 42) strawberry calli; bars represent standard deviations; (CCF = culture filtrate; CD = Czapek-Dox, control; data taken after 30 days).

Fig. 2. Response of susceptible ('Tangi') and resistant (MSUS 42) strawberry calli to culture filtrate of C. fragariae, isolate CF1. (CCF = culture filtrate, CD = Czapek-Dox, control; data taken after 30 days).



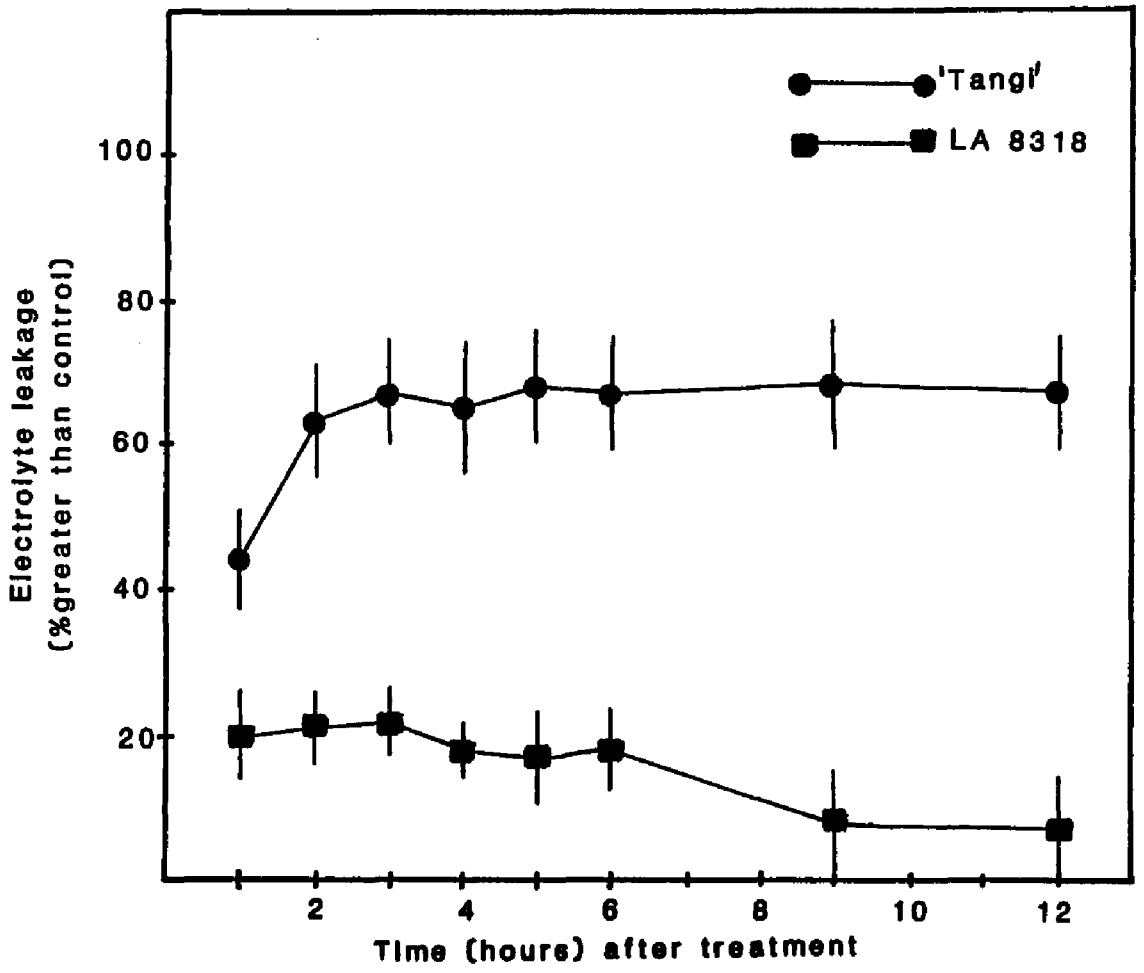


Fig. 3. Electrolyte leakage in susceptible ('Tangi') and resistant (LA 8318) strawberry calli treated with culture filtrate of C. fragariae, isolate CF1. Data represent means and standard deviations (6 replicates) of specific conductance (umhos/cm^3) of treated minus specific conductance of control, expressed as percentage of initial readings at time 0.

IN VITRO SCREENING FOR ANTHRACNOSE (*Colletotrichum fragariae* Brooks) RESISTANCE IN STRAWBERRY

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Abstract

Calli of 8 strawberry cultivars/lines responded differently when inoculated with conidial suspension of *Colletotrichum fragariae*. The fungus grew comparatively less on MSUS 42, LA 8318, and LA 6261 calli than on the susceptible check 'Tangi' and grew equally or more on LA 42311, 'Apollo', 'Sunrise', and 'Earlibelle'. Sporulation of the fungus was less on MSUS 42, LA 8318, LA 6261, and 'Apollo'. When subcultured on a medium containing 50% culture filtrate, MSUS 42, LA 8318, LA 6261, 'Apollo' and 'Earlibelle' calli were affected less by the culture filtrate. Ratings of in vitro screened callus tissues indicated that MSUS 42, LA 8318, LA 6261, and 'Apollo' were resistant, 'Earlibelle' was intermediate, and MSUS 42311, 'Tangi', and 'Sunrise' were susceptible. In vitro screening results correlate well (0.812) with whole plant level screening responses.

Abbreviations: BA: N-(phenyl-methyl)-1-H-purine-6-amine, IBA: 1H-indole-3-butanoic acid, GA_3: gibberellic acid, NAA: 1-naphthaleneacetic acid, MS: Murashige and Skoog [18].

INTRODUCTION

Crown rot, caused by Colletotrichum fragariae Brooks, is the most devastating phase of the anthracnose disease of strawberry. Screening for anthracnose resistance is normally accomplished by distal petiole inoculation [7], plant spray inoculation [21], or by crown injection [20]. These methods offer reliable and accurate results but require large space allocations to screen breeding lines.

Tissue culture as a research tool has been used in several host-parasite studies. Results obtained in tobacco x Phytophthora parasitica var. nicotianae [6, 11, 12], potato x P. infestans [3, 16], soybean x P. megasperma var. sojae [13], alfalfa x P. megasperma var. medicaginis [17], and tomato x P. infestans systems [23] demonstrated that disease resistance and susceptibility can be expressed in vitro. This opens up the possibility of utilizing tissue culture systems as simple models to study host-parasite relationships and the mechanism of resistance. The use of tobacco suspension cultures provided easy manipulation of environmental conditions in investigating the biochemical mechanism of hypersensitive reaction of tobacco to Pseudomonas syringae pv. pisi [1, 2]. Phytoalexin accumulation in pathogen-challenged tobacco callus tissues was related to the extent of colonization of P. parasitica pv. nicotianae [4].

The present trend of international germplasm exchange through tissue culture to minimize the danger of introducing pathogens to the recipient country can also be benefited if plant materials can be evaluated in vitro. Although ambitious, the practical implications cannot be over-emphasized. Ways by which genetic materials can be effectively screened in vitro will mean substantial savings on time, labor, and money as more materials can be handled within the confines of the laboratory.

The main objective of this study was to evaluate the disease response of 8 strawberry cultivars/lines using tissue culture techniques and to correlate the results with the whole plant level response.

MATERIALS AND METHODS

Establishment of Strawberry Tissue Cultures

Runners of 'Tangi', MSUS 42, LA 8318, LA 6261, and LA 42311 were obtained from Louisiana State University Agricultural Experiment Station in Baton Rouge, Louisiana while runners of 'Earlibelle', 'Apollo', and 'Sunrise' were purchased from Lewis Nursery (Rocky Point, North Carolina). Sterilization was accomplished by immersing the shoots for 10 minutes in a 1.05% sodium hypochlorite solution (20% commercial bleach solution) containing a drop of Tween 20. The shoots were rinsed twice with

sterile distilled water, the outer leaves were removed, and then the shoots were reimmersed in 0.52% sodium hypochlorite solution for 5 minutes, and then finally rinsed three times with sterile distilled water. The shoot tips were established in 25 x 150 mm culture tubes containing 10 mls modified MS medium supplemented with 1 mg/l BA, 1 mg/l IBA, 0.01 mg/l GA_3, 30 g/l sucrose, and 8 g/l agar. The pH of the medium was adjusted to 5.7 prior to autoclaving (1 kg/cm^2, 121°C for 15 minutes). Calli formed on the shoots were transferred onto another medium containing the basal MS salts supplemented with 1 mg/l NAA, 1 mg/l BA, 100 mg/l myo-inositol, 10 mg/l thiamine, 30 g/l sucrose and 8 g/l agar. All maintenance callus cultures were incubated at 25°C under continuous light (75 uE m^{-2} sec^{-1}).

Inoculation and Incubation

Culture of C. fragariae, isolate CF1, was obtained from Dr. Barbara Smith, USDA, Poplarville, Mississippi and was preserved in silica gel [19]. Fungal cultures were initiated by placing several silica crystals on petri plates containing a mixture of potato dextrose agar (19.5 g/l) and oatmeal agar (30 g/l). Fungal plates were incubated at 25°C under continuous cool white fluorescent light (100 uE m^{-2} sec^{-1}). Inoculum was prepared by flooding 10-14 day-old plates with sterile distilled

water, filtering through cheesecloth and diluting with water until the desired concentration was reached. Normally, 10 ul of the prepared suspension delivered 20-25 conidia.

Four-week old calli subcultured in scintillation vials containing 10 ml of callus culture medium were preincubated in the dark at 15^{o}C for 24 hours prior to inoculation with conidial suspension (20-25 conidia/callus) of the fungus. Inoculated calli were incubated in the dark at 15^{o}C and rated daily based on fungal colonization rating [17]: 0 = no aerial hyphae visible, 1 = aerial hyphae on upper 25% of callus, 2 = aerial hyphae on upper 50% of callus, 3 = aerial hyphae on upper 75% of callus , and 4 = aerial hyphae on entire callus. Sporulation was estimated by taking a portion of the mycelia (25 mm^{2}) from 3 representative samples from each treatment at the end of the experiment, triturating it in 0.5 ml distilled water and taking the spore count using a hemacytometer.

Ten calli per cultivar/line were inoculated. The experiment was repeated 3 times, each repetition serving as a replicate (block). Data were expressed as percentage of the susceptible check 'Tangi', transformed using arcsin transformation and analyzed using the transformed values. Cultivars/lines were compared with 'Tangi' using the Dunnet's test.

Preparation of Culture Filtrate

One-liter flasks containing 200 ml of sterilized liquid Czapek-Dox medium [22] were inoculated with 20 mycelial plugs (15 cm^2) from 7 to 10 day-old fungal cultures growing on potato dextrose agar and oatmeal agar. Inoculated flasks were incubated in the dark at 25°C for 3 to 4 weeks. The culture filtrate was filtered sequentially through several layers of cheesecloth, Whatman #42 filter paper, Seitz filter (0.45 u), and finally filter sterilized using the Nalgene filter unit (0.22 u) and stored in the freezer (-4°C) until needed.

Callus culture medium containing 50% culture filtrate was prepared by replacing water with filter sterilized culture filtrate that was added to the autoclaved medium when it had cooled down to 35°C. Similarly stored uninoculated Czapek-Dox liquid medium was used as control. Fifteen ml of the prepared medium were dispensed into petri plates and inoculated with callus of the 8 strawberry cultivars/lines. Four plates containing 4 calli each were used per cultivar/line. The petri plates were incubated at 25°C under continuous cool white fluorescent light (75 uE m^{-2} sec^{-1}). Calli were observed for any sign of discoloration for 30 days. The experiment was repeated twice using separate batches of culture filtrate.

Whole Plant Level Evaluation

Nine plants of each cultivar/line were spray inoculated with conidial suspension of C. fragariae, isolate CF1 (10^6 conidia/ml). Four month old plants growing in 10 cm plastic pots were used in this experiment. Inoculated plants were incubated in a mist chamber at 100% relative humidity at 32-35°C for 48 hours. Plants were transferred to the greenhouse (day temperature: 32-35°C; night temperature: 25-28°C). Disease severity ratings were taken after 30 days using Smith's [20] rating system: 0 = healthy plants with no visible lesions, 1 = plant with petiole lesions <3 mm long, 2 = plant with petiole lesions 3-10 mm long, 3 = plant with petiole lesions 10-20 mm long usually girdling the petiole, 4 = plant with petiole lesions >20 mm to entire petiole necrotic, 5 = plant where youngest leaf was wilted indicating crown infection with or without petiole lesion, 6 = plant dead, crown necrotic.

Scores were averaged and each cultivar/line was classified according to Smith's [20] suggested groupings: 0-2 = resistant, 2.1-3.9 = intermediate, and 4-6 = susceptible.

RESULTS

Fungal Growth

C. fragariae, isolate CF1, exhibited differential growth when inoculated on calli of 8 strawberry cultivars/lines (Tables 1 and 2). Earlier results indicated that the best differential can be observed on the seventh day of incubation at 15°C. At this time, MSUS 42, LA 8318, and LA 6261 received significantly lower fungal growth ratings than the control cultivar ('Tangi') and the rest of the cultivars/lines tested. Dunnet's test revealed that these strawberry lines were less susceptible than 'Tangi', possibly by limiting the growth of the pathogen.

Sporulation

Sporulation of C. fragariae, isolate CF1, varied on calli of 8 strawberry cultivars/lines. The lowest sporulation was detected on LA 8318 and 'Apollo' with a value of 58% less than that on 'Tangi'. MSUS 42 and LA 6261 had 47 and 26%, less sporulation, respectively, than 'Tangi' (Table 1). Based on Dunnet's test, any cultivar/line having more than 17% reduction in sporulation can be considered more resistant than 'Tangi', thus, LA 8318, MSUS 42, and 'Apollo' were rated as resistant, LA 6261 intermediate, and 'Earlibelle' and LA 42311 susceptible.

Response of Strawberry Calli to Culture Filtrate

Calli of 8 strawberry cultivars/lines subcultured on a medium containing 50% culture filtrate exhibited differing degrees of susceptibility to the culture filtrate enriched medium. 'Tangi', 'Sunrise', and LA 42311 calli became necrotic within the first week on the medium containing culture filtrate and were dark brown throughout and exhibited no sign of growth after 30 days (Fig. 1). Conversely, MSUS 42, LA 8318, LA 6261, 'Apollo', and 'Earlibelle' exhibited minimal discoloration during the first week and had substantial growth over the 30-day observation period. This observation may indicate the different levels of sensitivity of the calli to certain components of the culture filtrate.

Whole Plant Level Evaluation

Disease severity ratings of the 8 strawberry cultivars/lines 30 days after spray inoculation with conidial suspension of the fungus (Table 3) indicated that MSUS 42 and LA 8318 were resistant, 'Apollo', LA 42311 and LA 6261 were intermediate, and 'Sunrise', 'Earlibelle', and 'Tangi' were susceptible. Results of whole plant level evaluation were in agreement and at times in contrast to published disease reactions of the different cultivars/lines. 'Apollo' which was rated

intermediate in this study was rated resistant by Smith [20] and susceptible by Delp and Milholland [8]. 'Sunrise' was previously classified both as resistant [8] and intermediate [20]. We found 'Earlibelle' to be susceptible but it was reported to be resistant [8]. 'Earlibelle' was one of the parents used in developing 'Florida Belle' because of its known resistance to anthracnose but it was later found to be susceptible [14]. The differences on the ratings given to 'Apollo', 'Sunrise', and 'Earlibelle' may be attributed to the higher incubation temperature in the greenhouse that averaged 32-35 ^{o}C during the day and 25-28^{o}C during the night. However, these temperatures are not uncommon for field conditions in Louisiana during the strawberry production cycle.

DISCUSSION

Results of in vitro selection techniques using artificial inoculation of calli with conidial suspension and incorporation of the culture filtrate into the medium showed that calli derived from 8 strawberry cultivars/lines responded differently to the applied treatments. Based on fungal growth ratings, MSUS 42, LA 8318, and LA 6261 expressed their resistance by limiting the growth of the pathogen. Reduced colonization of the resistant tissues by the pathogen has been reported in

alfalfa [17], tobacco [11, 12], tomato [23], potato [16], and soybean [13]. Sporulation data also indicated the capacity of the three above mentioned lines and 'Apollo' to check the multiplication of the fungus. Although fungal growth on 'Apollo' was not statistically different from that on 'Tangi', this cultivar expressed its resistance by drastically reducing the sporulation rate of the fungus. Similarly, in the tobacco callus inoculated with Pseudomonas tabaci, the bacteria multiplied rapidly while P. pisi and P. fluorescens (both nonpathogens) multiplied comparatively less [15]. The response of the different calli when exposed to the culture filtrate also suggested the ability of MSUS 42, LA 8318, LA 6261, 'Apollo', and 'Earlibelle' to neutralize or metabolize whatever toxic components were present in the culture filtrate. It could also indicate that the binding sites for the interaction with the toxin were unavailable or absent [9] or the toxin may have activated the self repair mechanism in the resistant tissues [15]. 'Earlibelle' callus received susceptible ratings based on fungal colonization and sporulation but its failure to exhibit discoloration when exposed to culture filtrate may qualify it in the intermediate category. 'Tangi', 'Sunrise', and LA 42311 consistently scored as susceptible while MSUS 42, LA 8318, and LA 6261 consistently scored as resistant.

A highly significant correlation coefficient of 0.812 was obtained when in vitro and greenhouse results were compared using Spearman's ranking correlation [5] (Table 3). Similarly, a very high correlation coefficient (0.971) between in vitro and greenhouse results was reported by Hartman et al. [10] when they tested the response of bean calli to the culture filtrate of P. syringae pv. phaseolicola. This would then indicate that in vitro results parallel those at the whole plant level. However, caution should be exercised in interpreting these results. Not all resistance mechanisms can be expressed in vitro and there is always the possibility of not detecting them in vitro, especially if such resistance mechanisms are in a form of a physical barrier (i.e. cutin). Moreover, resistance genes may be present but they may not be actively transcribed and translated leading to erroneous interpretations. Results obtained from this study can be meaningful if it can be shown that the mechanisms of resistance at the whole plant level are due to factors that limit the growth and multiplication of the fungus and the ability of the resistant plants to metabolize any toxic compounds that the fungus produces. Isolation, purification, and assay of the toxic components of the culture filtrate are necessary. Investigations along these lines need to be pursued.

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Table 1. Response of 8 strawberry cultivars/lines to some in vitro selection methods to identify anthracnose resistant cell lines.

Cultivar/ Line	Fungal growth rating[z]	Sporulation[y]	Culture filtrate induced discoloration[x]
'Tangi'	100 [S]	100 [S]	+ [S]
'Apollo'	95 (15) [S]	42 (10) [R]	- [R]
'Sunrise'	89 (10) [S]	93 (24) [S]	+ [S]
'Earlibelle'	91 (9) [S]	97 (18) [S]	- [R]
LA 42311	93 (7) [S]	143 (40) [S]	+ [S]
LA 8318	63 (10) [R]	42 (6) [R]	- [R]
LA 6261	58 (8) [R]	74 (6) [I]	- [R]
MSUS 42	68 (10) [R]	53 (13) [R]	- [R]

[z]Expressed as percentage of 'Tangi'; significant ($P<0.1$); Dunnet's critical value = 19%; values in parentheses are standard deviations; data taken 7 days after inoculation; incubation temperature = 15°C.

[y]Expressed as percentage of 'Tangi'; highly significant ($P<0.01$); Dunnet's critical value = 17%; values in parentheses are standard deviations.

[x]Data taken 30 days after subculture in a medium containing 50% culture filtrate.

Table 2. Growth and sporulation of C. fragariae, isolate CF1, on calli of 8 strawberry cultivars/lines[z].

Cultivar/line	Fungal growth rating[y]	Spore count[x] (10^3)
'Tangi'	1.87 (0.10)	14.17 (2.33)
'Apollo'	1.77 (0.29)	5.35 (0.71)
'Sunrise'	1.63 (0.13)	13.47 (4.32)
'Earlibelle'	1.70 (0.18)	12.71 (0.85)
LA 42311	1.77 (0.38)	18.96 (5.42)
LA 8318	1.20 (0.26)	6.32 (1.82)
LA 6261	1.10 (0.17)	10.14 (1.25)
MSUS 42	1.30 (0.26)	7.43 (2.42)

[z]Incubation temperature, 15°C.

[y]0-4 rating system[17]: 0 = no aerial hyphae visible, 4 = aerial hyphae on entire callus; data taken 7 days after inoculation; average of 3 replications with 10 calli per replication; values in parentheses represent standard deviations.

[x]Average of 3 replications with 3 random samples per treatment; estimated by triturating 25 mm^2 mycelia in 0.5 ml water and making a spore count using a hemacytometer; values in parentheses represent standard deviations.

Table 3. Correlation between greenhouse disease response and in vitro disease response of 8 strawberry cultivars/lines to C. fragariae[z].

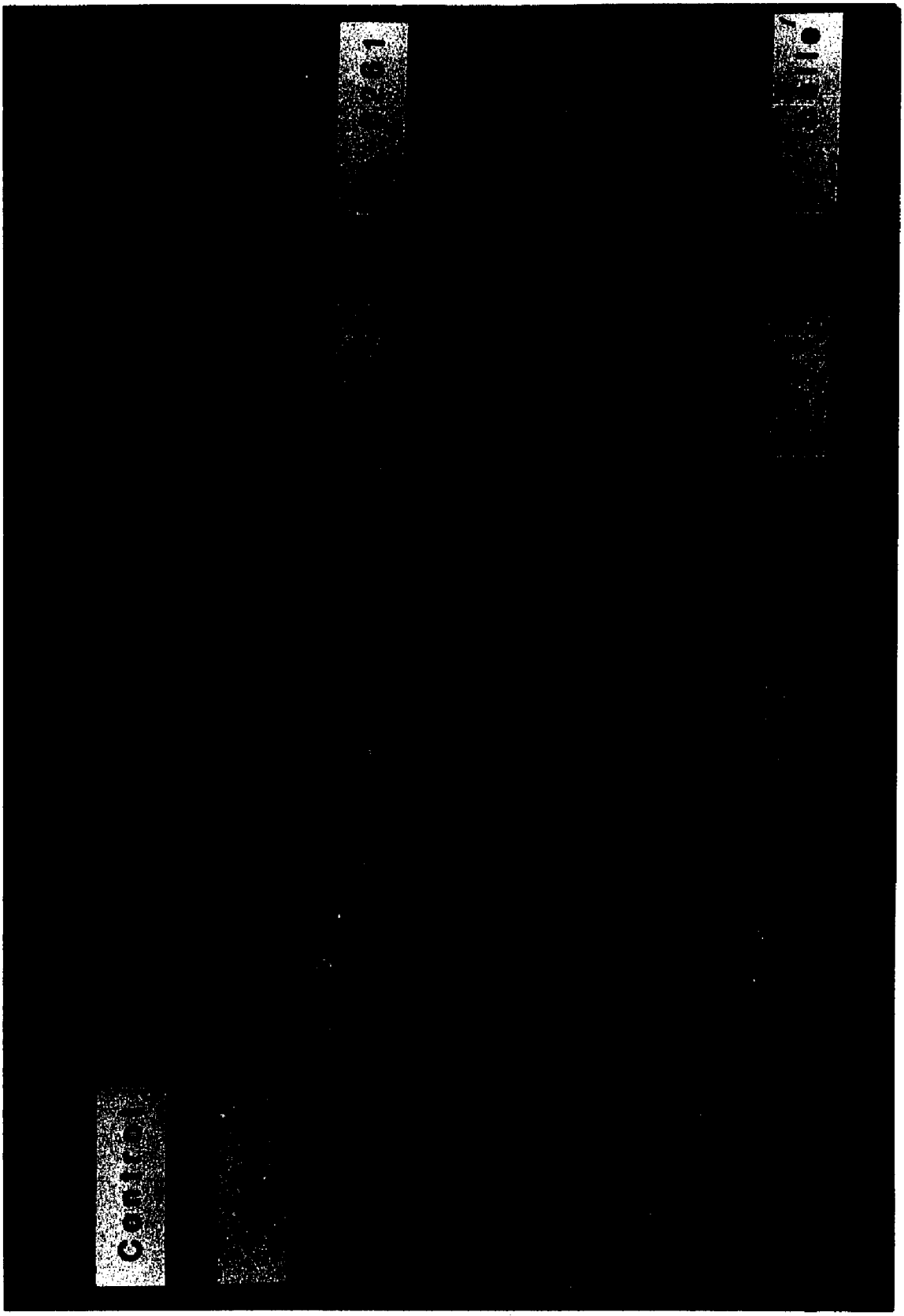
Cultivar/Line	Overall in vitro disease response[y]	Greenhouse disease response[x]
'Tangi'	3.0	5.7
'Apollo'	1.6	3.3
'Sunrise'	3.0	5.7
'Earlibelle'	2.3	6.0
LA 42311	3.0	3.8
LA 8318	1.0	1.2
LA 6261	1.3	3.5
MSUS 42	1.0	1.2

[z]Spearman's [5] correlation ranking coefficient = 0.812 (highly significant, $P<0.01$).

[y]Averaged response based on fungal growth rating, sporulation, and reaction to culture filtrate (1 = resistant, 3 = susceptible).

[x]Based on Smith's [19] rating system: 0-2 = resistant, 2.1-3.9 = intermediate, 4.0-6.0 = susceptible; mean score of 9 plants; data collected 30 days after inoculation.

Fig. 1. Response of calli derived from 8 strawberry cultivars/lines to culture filtrate of C. fragariae, isolate CF1. (CF = 50% culture filtrate; Control = 50% Czapek-Dox medium; data taken after 30 days).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VITA

Violeta Nano Villegas was born on January 13, 1954 in Taal, Batangas, Philippines as the fifth child in a family of 8 girls. She was raised in Calamba, Laguna, Philippines where she attended both public and private schools for her elementary and high school education. She obtained both her BS in Agriculture (major in Horticulture, minor in Soil Science) and MS in Horticulture (major in plant breeding and genetics, minor in physiology) degrees in 1975 and 1979, respectively, from the University of the Philippines at Los Banos (UPLB). She has been working with the same university since 1975, doing fruit breeding related research and teaching courses in horticulture, plant breeding, and genetics. While on study leave from her position as a Science Research Specialist at the Institute of Plant Breeding, UPLB, she attended the Louisiana State University from 1983 to 1986. Her anticipated date of graduation is December, 1986. She will assume a research/teaching position when she returns to her country.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Horticulture

Title of Dissertation: Use of Tissue Culture Methods in Screening for Anthracnose (Colletotrichum fragariae Brooks) Resistance in Strawberry

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